OXIDATIVE STRESS ASSESSED IN SALIVA FROM PATIENTS WITH ACUTE MYOCARDIAL INFARCTION. A PRELIMINARY STUDY

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ABSTRACT

There is evidence that acute myocardial infarction (AMI) is associated with increasing production of reactive oxygen species and tissue injury. The aim of this study was to assess the presence of oxidative stress indices in saliva 24 and 48h after AMI. Materials and methods: We designed a prospective study comparing salivary levels of biomarkers of oxidative stress in patients with AMI with elevation of the ST segment in electrocardiogram versus clinically healthy subjects. Oxidative stress indices including the rate of oxidation of 2'7' dichlorohydrofluorescein diacetate (DCFH-DA) and the activity of the antioxidant enzyme catalase (CAT) were evaluated in saliva from patients with AMI at 24 and 48 hours. At each sampling time, blood was drawn for serum markers of myocardial infarction. Results: This study included ten patients with acute ST-segment elevation myocardial infarction and ten clinically healthy controls. Mean age was 67.8 ± 11.1 vs. 48.7 ± 4.1 years ($p<0.001$) and gender was 60% male vs. 50% ($p>0.05$) for AMI vs. controls, respectively. Our results demonstrated an increase in the rate of oxidation of DCFH-DA in the myocardial infarction group as compared with controls ($p=0.004$), which remained unchanged at 48h. There was no difference in salivary catalase activity between controls and AMI subjects at 24h or at 48h post-diagnosis ($p=0.157$). The relationship between CAT_{48} and $DCFH\text{-}DA_{48}$ was fairly significant ($r=0.39$; $p=0.053$). Conclusion: This preliminary study showed that biomarkers of oxidative stress are detectable in saliva of patients with acute myocardial infarction. Clinical Relevance: Future studies using a larger population are needed to confirm these observations and to explore the possibility of using the saliva to monitor evolving diagnosis and prognosis in acute coronary syndrome.

Key Words: *saliva, acute myocardial infarction, acute coronary syndrome, dichlorohydrofluorescein diacetate, catalase, oxidative stress.*

MARCADORES DE ESTRES OXIDATIVO EN SALIVA DE PACIENTES CON INFARTO AGUDO DE MIOCARDIO. ESTUDIO PRELIMINAR

RESUMEN

Existe evidencia que permite establecer una asociación entre la generación de especies reactivas del oxígeno y el daño tisular en el síndrome coronario agudo. El objetivo de este trabajo fue detectar en saliva de pacientes con infarto agudo de miocardio (IAM), la presencia de reactantes de estrés oxidativo a las 24 y 48 horas. Materiales y métodos: se efectuó un estudio prospectivo de comparación entre pacientes con IAM con supradesnivel del segmento ST en el electrocardiograma y sujetos sin patología clínica evidente. La producción de especies reactivas de oxígeno fue evaluada mediante la tasa de oxidación de la 2'7' diacetato de diclorohidrofluoreceina (DCFH-DA) y la actividad antioxidante de la enzima catalasa (CAT) en saliva de pacientes con IAM a las 24 y 48 h de producido el síndrome coronario agudo. Simultáneamente, se determinaron en suero los biomarcadores diagnósticos de IAM. Resultados: se incorporaron 10 pacientes con IAM con supradesnivel del ST que fueron comparados con 10 sujetos del grupo control. La edad promedio fue 67.8 ± 11.1 vs 48.7 ± 4.1 años, respectivamente ($p<0.001$); el 60% vs 50% fueron hombres sin diferencias entre ambos grupos ($p>0.05$). La media de la velocidad de oxidación de la DCFH-DA fue mayor a las 24 h en los pacientes con IAM ($p=0.004$). Estas diferencias se mantuvieron a las 48 h del infarto sin cambios significativos. No se encontraron diferencias en las medias de actividad de la enzima catalasa entre IAM y control ($p>0.05$). Se encontró una relación entre CAT_{48} y $DCFH\text{-}DA_{48}$ ($r=0.39$; $p=0.053$). Conclusiones: En esta población se han detectado reactantes de estrés oxidativo en saliva de pacientes con IAM. Relevancia clínica: nuevos estudios con mayor número de casos serán necesarios para confirmar estas observaciones y evaluar la utilidad de la saliva en el diagnóstico, evolución y pronóstico del síndrome coronario agudo.

Palabras clave: *saliva, infarto agudo de miocardio, síndrome coronario agudo, diacetato diclorohidrofluoreceina, catalasa, estrés oxidativo.*

INTRODUCTION

Reactive oxygen species (ROS) are oxidants/reductants and mainly regarded as hazardous species whose production in cellular and extracellular systems has to be tightly controlled by antioxidants and radical scavenging biochemical reactions. Recently, the importance of radical species in cellular signaling and in the maintenance of homeostatic conditions has been rec-

ognized. The toxicity of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) arises from the Fe-dependent conversion into the extremely reactive hydroxyl radical ($^{\cdot}OH$) (Haber Weiss reaction) that causes severe damage to membranes, proteins, and DNA. Arguably, some radicals, such as the very short-lived and extremely hazardous $^{\cdot}OH$, are still regarded as highly reactive and dangerous, but many other more stable species have been postulated as signaling molecules for cellular growth or as oxidants that ensure an appropriate oxidation state of cellular compartments and the biochemical structures and elements they contain. According to the present view, a basal amount of ROS is formed at all times in all aerobic cells, and the steady state concentration of ROS in each cell or compartment depends on the formation rate of the radical, its reactivity and the concentration of available reaction partners. Indeed, the excessive production of free radicals overwhelms the available tissue defense, resulting in the destruction of the tissues by the generation of oxidative stress, as a result of an imbalance between ROS production and the endogenous antioxidant mechanisms to neutralize their effects[1].

Among a long list of pathologies and diseases, including carcinoma[2] and type 2 diabetes[3], strong evidence suggested that ROS may play an important role in the pathogenesis of myocardial infarction. Moreover, endothelial dysfunction is characterized by reduced nitric oxide (NO) bioavailability and increased generation of ROS in the vascular wall[4]. However, beneficial effects in terms of myocardial salvage reperfusion itself may contribute to additional damage of the myocardium, due to the combined processes known as "ischemia-reperfusion injury". ROS are known to be produced in large quantities post-ischemia reperfusion process[5], leading to additional myocardial injury beyond that generated by ischemia itself.

Previous studies performed on patients with myocardial disease showed that biomarkers of oxidative stress were significantly higher in serum[6-8], and in myocytes [9,10].

Among the biological fluids, it was reported that components of saliva can serve as biomarkers not only for oral disorders [11,12], but for different pathologies including osteoporosis, cancer, HIV, and autoimmune, viral, bacterial, and cardiovascular diseases as well [13,14]. The hypothesis of this work is that acute myocardial infarction (AMI) generates oxidative stress detectable in saliva. In this study, the production of ROS, assessed by the rate of oxidation of 2'7' dichlorohydrofluorescein diacetate (DCFH-DA), and the activity of the antioxidant enzyme catalase (CAT) were evaluated in saliva from patients with AMI and control subjects.

MATERIALS AND METHODS

Subject selection and study design

We performed a prospective comparative study between patients with AMI and patients without clinical evidence of cardiovascular disease or other known disease (healthy controls). AMI was characterized by laboratory, clinical and electrocardiographic criteria. The diagnosis was defined by increased serum creatine phosphokinase (CPK; males >190 IU/l, females >170 IU/l) and troponin T (TnT >3 ng/ml) concentrations, in addition to chest pain for more than 20 min at rest and ST-segment elevation at least in two contiguous leads in electrocardiography. Informed consent was obtained from all participants prior to their enrollment in the study. The protocol was approved by the local Ethics Committee.

Both 24 (AMI_{24}) and 48h (AMI_{48}) after the coronary event, blood samples were collected, immediately processed (centrifuged at 2000*g* for 15 min), for CPK (Cobas c311, Roche) and TnT (Cobas e411, Roche); aliquots were stored at minus 40°C to determine C-Reactive Protein (CRP; normal value <8 mg/l) by a turbidimetric immunoassay (InCCA, Diagam).

At each time sampling unstimulated saliva was collected in specific tubes for routine biochemistry analysis. Samples were immediately centrifuged and stored in 0.5 ml aliquots at -40ºC. The ability to generate ROS was detected as the rate of oxidation of DCFH-DA, as previously reported by González et al. [15] The reaction was followed in a 30 mM HEPES, pH 7.2 buffer, with 200 mM KCl and 1 mM $MgCl_2$. The fluorescent probe DCFH-DA was added to the buffer, in a final concentration of 40 μM, along with an aliquot of saliva previously centrifuged for 20 min at 2000*g* for clarification of the sample. The reaction mixture was incubated at 37ºC for 20 min and fluorescence was detected spectrofluorometrically at λ_{ex}=488 nm and λ_{em}=525 nm.

CAT activity (EC 1.11.1.6) was assayed spectrophotometrically by the decomposition of H_2O_2 at λ=240 nm in a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2 [16]. Protein measurements in saliva samples were performed according to Lowry et al. [17].

Statistical analysis

Data were analyzed by the statistical package SPSS 16, One-Way Analysis of Variance (ANOVA) Kolmogorov-Smirnov, Linear regression and Spearman correlation coefficients were included. Confidence intervals at 95% and α=0.05 were set.

RESULTS

This study assessed 50 consecutive adult patients with acute coronary event admitted to the Cardiology Division, Hospital Español, Buenos Aires, Argentina, from June 2012 to November 2012. Of those 50 patients, 10 (20%) were diagnosed with AMI. Included patients were all diagnosed with AMI with ST elevation at least in two contiguous leads. Ten healthy subjects were selected as controls. Mean age was 67.8 ± 11.1 vs. 48.7 ± 4.1 years (p<0.001) and 60% vs. 50% were male (p> *0.05)* for AMI vs. controls respectively.

The coronary angiography revealed coronary occlusion in the anterior descending artery (60%), in the right coronary artery (30%) and in the circumflex artery (10%) and showed severe lesions that involved 3 (10%), 2 (20%) or 1 (60%) coronary arteries; 10% of patients had affection of the left coronary artery and three coronary vessels.

At 24 and 48h, patients with AMI exhibited significantly higher serum CPK (1699.3 ± 1049.5 and 1098.5 ± 713.3 vs. 186 ± 103 IU/l; p<0.0001) and TnT (3.8 ± 1.3 and 3.3 ± 1.5 vs. 0.12 ± 0.05 ng/ml; p<0.0001) concentrations vs. controls. Serum CRP revealed increased levels as compared with controls (36.0 ± 40.7 vs. 4.4 ± 1.1 mg/l; p=0.02).

DCFH-DA oxidation rate by patient saliva was evaluated as an index for the chemical ROS generation capacity. The fluorescent compound DCF, generated by radical-dependent oxidation of the probe, was detected. At 24h, AMI group demonstrated significantly higher salivary DCFH-DA levels as compared to healthy individuals (p=0.03); which remained unchanged at 48h (Fig.1A). There was no difference in salivary CAT activity between patients with AMI and healthy subjects (p=0.157) (Fig.1B).

The correlation analysis revealed a no significant correlation between salivary CAT_{24} and $DCFH\text{-}DA_{24}$ levels (p=0.293) (Fig.2A). However, a fairly significant correlation was found at 48h (r*=0.39;* p=0.053) (Fig.2B).

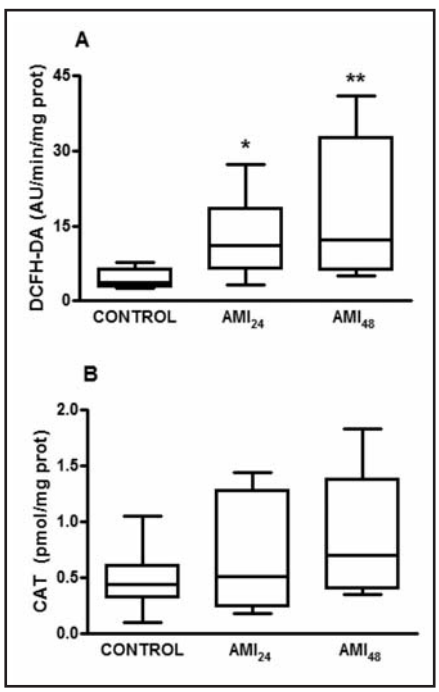

Fig. 1: Oxidative stress indices in saliva of patients with acute myocardial infarction. AMI: acute myocardial infarction; control: healthy subjects; DCFH-DA: 2'7' dichlorohydrofluorescein diacetate; CAT: catalase activity. Salivary concentrations of : A. DCFH-DA levels and B. CAT activity in AMI patients (at 24h, AMI_{24} and 48h, AMI_{48} post diagnosis) and healthy subjects. Minimum and maximum values are indicated by bottom and topmost points of box plot. Lower quartile, median and upper quartile values are indicated by first, second and third horizontal lines of the box in the box plot. Significance is shown as * p<0.05, ** p<0.01. A. * p<0.05, AMI_{24} vs. Control; ** p<0.01, AMI_{48} vs. Control; p>0.05, AMI_{24} vs. AMI_{48}. B. p>0.05, AMI_{24} vs. AMI_{48} vs. Control.

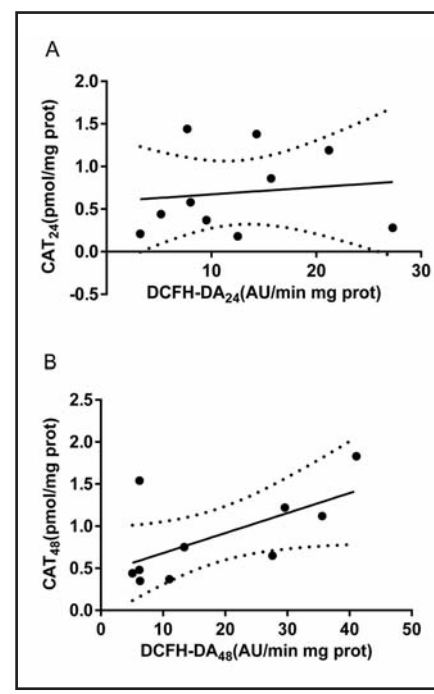

Fig. 2: Relationship between salivary CAT activity and DCFH-DA concentrations in acute myocardial infarction patients at 24h and 48h post cardiac event. AMI: acute myocardial infarction; CAT: catalase activity; DCFH-DA: 2'7' dichlorohydrofluorescein diacetate; A. no significant correlation was found at 24h (p=0.293).B. a fairly significant correlation was found at 48h (r=0.39; p=0.053).

DISCUSSION

Saliva was proposed as a potential diagnostic medium for several pathologies [18,19]. Moreover, salivary assays, unlike determinations in blood, are less invasive, and can be self-administered without special equipment or personnel [11]. The potential early changes in biochemical biomarkers in saliva could provide valuable insights with the advantage of being an easy, safe, cost-effective, and noninvasive diagnostic approach.

In this study, the total intracellular ROS generation, assessed in saliva by the oxidation of DCFH-DA, was significantly increased in AMI patients compared to control subjects. This result is consistent with recent findings successfully applying this methodology in myocytes of AMI patients [9,10], which showed an increase in ROS levels. H_2O_2 has been described as one of the oxidants responsible for DCFH-DA oxidation along with several others [20]. The mechanism proposed by King et al. [21] considered the generation of H_2O_2, as shown in reaction 2 in the presence of Fe and O_2 (reactions 1 and 2).

(1) $Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^-$

(2) $2H+ + Fe^{2+} + O_2^- \longrightarrow Fe^{3+} + H_2O_2$

H_2O_2 is a good candidate for triggering cellular responses since it is the most stable of the reactive intermediates of O_2 reduction [22]. H_2O_2 diffuses freely into the tissue and increases the oxidative stress (measured as DCFHDA-oxidation) and further causes oxidative damage. H_2O_2 is especially toxic through the Fenton reaction with Fe^{2+}, where it gives rise to the extremely reactive $^{\cdot}OH$ (reaction 3).

(3) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + {}^{\cdot}OH$

Thus, H_2O_2-induced oxidative stress may trigger the endogenous antioxidant system. Similar to findings reported by others[23], the present study showed that patients with acute myocardial infarction had increased serum levels of oxidative stress associated with a reduction in the enzymatic antioxidant reserve, since there was no significant increase in catalase activity. Increased levels of free radical generating system and no change in free radical scavenging systems lead to generation of oxidative stress that may be critical in ischaemic heart conditions. The overproduction of ROS during AMI could overwhelm endogenous scavengers (antioxidants), thus leading to cell death.

By 48h, the positive relationship between salivary DCFH-DA and CAT levels in AMI patients suggests a tendency to achieve a balance between pro-oxidants and antioxidants.

In this preliminary study, the age difference was expected since coronary diseases are more prone to occur in elderly people.

Future studies are planned to demonstrate the dynamics of the antioxidants adjusting the pathophysiological conditions imposed.

These results show that the sustained oxidative stress induced by AMI was reflected by the increases in DCFH-DA oxidation and CAT activity, which are detectable in saliva.

In conclusion, biomarkers of oxidative stress in saliva could be analytes of easy access for the diagnosis and follow-up acute myocardial infarction. However, further studies are needed using a larger population to confirm these observations and explore the possibility of using saliva for diagnosis, evolution and prognosis in acute coronary syndrome.

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